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Integrating Chemistry and Mechanics: The Forces Driving Axon Growth

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Abstract

The brain is our most complex organ. During development, neurons extend axons, which may grow over long distances along well-defined pathways to connect to distant targets. Our current understanding of axon pathfinding is largely based on chemical signaling by attractive and repulsive guidance cues. These cues instruct motile growth cones, the leading tips of growing axons, where to turn and where to stop. However, it is not chemical signals that cause motion—motion is driven by forces. Yet our current understanding of the mechanical regulation of axon growth is very limited. In this review, I discuss the origin of the cellular forces controlling axon growth and pathfinding, and how mechanical signals encountered by growing axons may be integrated with chemical signals. This mechanochemical cross talk is an important but often overlooked aspect of cell motility that has major implications for many physiological and pathological processes involving neuronal growth.

Contents

1. INTRODUCTION

Neurons are among the most complex cells in the animal kingdom. They are highly polar, with typically several shorter, branched dendrites and one long axon emanating from the cell body. During development, neurons migrate to their final position and extend axons over long distances along well-defined pathways. At the tip of the advancing axon, a highly motile structure termed the growth cone (GC) is responsible for detecting environmental signals, adapting axon growth to them, and finding the proper postsynaptic target. Most of our knowledge about how GCs sense where to grow is based on chemical signaling: A diverse set of attractive and repulsive guidance cues directs growing neuronal axons to their final destination (Goldberg 2003, Gomez & Letourneau 2014, Holt & Harris 1993, Song & Poo 2001, Tessier-Lavigne & Goodman 1996).

However, these chemical guidance signals are not what causes GCs and axons to move. According to Newton's first law, motion is driven by forces, and hence axons must exert mechanical forces on their environment in order to move (Bray 1979, Heidemann et al. 1990, Lamoureux et al. 1989, Miller & Suter 2018, Suter & Miller 2011). Without these intrinsic forces, an axon would not move anywhere, even in the presence of strong gradients of chemical guidance cues. As an analogy, consider the smell of coffee in the morning. While this smell may be a strong attractant, we will not get anywhere near the coffee unless we exert some force on our environment by moving our legs.

Nevertheless, in comparison to the chemical regulation of neuronal growth, how chemical signals are translated into mechanical forces and how neurons mechanically interact with their environment are currently much less well understood. In addition, although a critical role of mechanical signals in regulating axon pathfinding was already suggested about a century ago (Harrison 1910, His 1887, Weiss 1934), we still know very little about it. Here I review work from the last four decades illuminating the role of mechanical forces in axon growth and develop a simple force-based toy model of axon growth and pathfinding that can explain most current experimental findings.

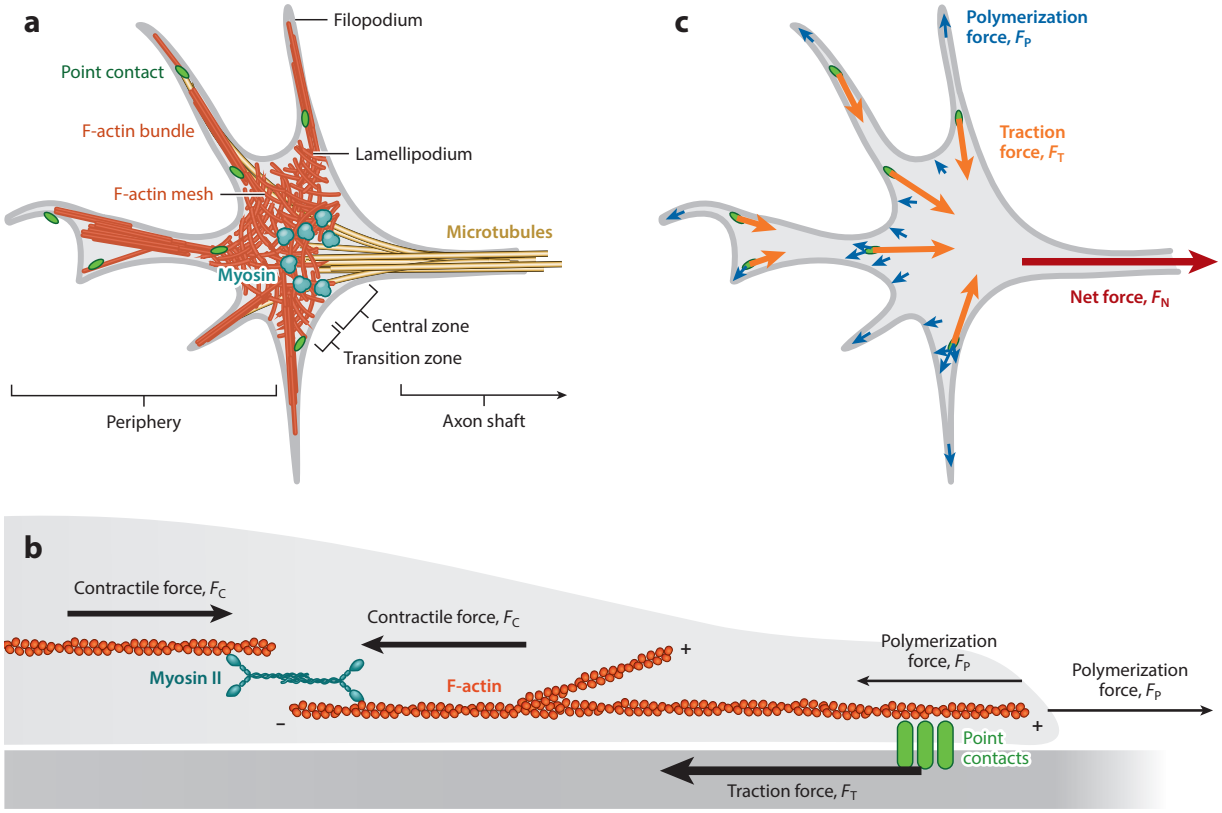

Figure 1

Origin of forces in neuronal growth cones (GCs). (*a*) Schematic drawing of a neuronal GC and its cytoskeleton. Filopodia, which are fingerlike protrusions packed with F-actin bundles, emanate from a flat lamellipodium, which mostly consists of an F-actin meshwork. Actin fibers are connected to the extracellular environment via molecular clutches, which are assembled in point contacts, the equivalent of comparatively larger focal adhesions found in other animal tissue cell types. Point contacts are found mainly in the GC periphery, while myosin II motors are localized at the GC central domain. Microtubules may splay in from the axon and invade individual filopodia. (*b*) Schematic drawing of force generation within GCs. Actin polymerization in the periphery leads to compressive polymerization forces, F_P, pushing actin against the cell membrane. At the same time, contractile forces, F_C, generated by myosin motors in the GC center pull actin filaments in the retrograde direction. The coupling of actin filaments with the extracellular environment through molecular clutches assembled in point contacts leads to the transmission of the internal forces to the outside. The resulting traction forces, F_T, pull on the substrate and so pull the GC base forward, thus generating space into which axonal microtubules can grow. (*c*) Schematic drawing of forces acting in GCs. If F-actin is coupled to the point contacts (i.e., if the molecular clutches are engaged), actin polymerization forces, F_P, push the membrane forward, while traction forces, F_T, generated by myosin motors pull on the substrate. Adding up all of the traction forces results in a nonzero net force, F_N, which pulls the GC base toward the point contacts, leading to tension along the axon. Larger arrows correspond to stronger forces. GC top view based on original illustration by Sarah Foster.

2. FORCES DRIVING GROWTH CONE MOTILITY

The forces required for GC motility and axon growth are generated by the neuronal cytoskeleton, a dense network of different polymers, mostly actin filaments (F-actin) and microtubules and their binding partners. To understand the individual contributions to the force balance that determines axon growth, I first consider GCs and axons separately.

GCs consist of a central domain, a flat lamellipodium, and fingerlike protrusions called filopodia (**Figure 1*a***). The morphology and mode of motility of GC lamellipodia are similar to those

found in other tissue cell types (Gardel et al. 2010, Giannone et al. 2009, Parsons et al. 2010). Lamellipodia are packed with actin filaments, which build dense, branched meshworks. The growing barbed ends of actin filaments point outward (i.e., toward the GC periphery) and polymerize against the GC membrane. Due to Brownian motion, both the actin filaments and the GC membrane fluctuate, thus occasionally leaving enough space for new actin monomers to be added. When the elongated actin filaments then move back to their initial configuration (i.e., when they straighten again due to fluctuations), they push against the membrane like a thermal ratchet (Peskin et al. 1993), putting the membrane under mechanical tension. The resulting polymerization force, F_P, of vertebrate neurons is on the order of ∼10 pN/μm (Amin et al. 2013, Cojoc et al. 2007), about 14 orders of magnitude less force than that exerted by gravity on us.

According to Newton's third law, the membrane simultaneously exerts a force equal in magnitude and opposite in direction on the actin filaments. Hence, polymerizing actin filaments are pushed backward (i.e., toward the GC center) (**Figure 1*c***) and actin's polymerization force is balanced by membrane tension of a similar magnitude (Hochmuth et al. 1996, Krieg et al. 2014). At the same time, myosin II motors, which are localized at the GC center, pull on the actin filaments (**Figure 1*c***). Without immobilization of actin filaments, these myosin-mediated contractile forces, F_C, together with polymerization forces, lead to constant retrograde (i.e., away from the GC periphery toward its center) flow of actin filaments (Craig et al. 2012, Lin et al. 1996, Medeiros et al. 2006). In this case, the energy required to generate these forces is dissipated by viscous flow, and the GC does not move forward.

However, actin filaments in the GC may be coupled to the extracellular environment via so-called point contacts (Nichol et al. 2016, Renaudin et al. 1999, Shimada et al. 2008, Suter et al. 1998), which are neuron-specific protein complexes similar to but usually smaller than the focal adhesions found in other tissue cell types. Point contacts consist of a large number of (mechanosensitive) adaptor and signaling proteins such as cell-adhesion molecules (CAMs), including integrins, talin, vinculin, shootin1, and cortactin (Case & Waterman 2015, Kubo et al. 2015, Renaudin et al. 1999, Suter et al. 1998, Toriyama et al. 2013). They dynamically connect the actin cytoskeleton with the extracellular matrix (ECM) and serve as molecular clutches (Mitchison & Kirschner 1988, Suter & Forscher 2000). The stronger this clutch is engaged, the stronger the actin is coupled to the outside world. In such molecular clutches, the strength of the link between actin and CAMs depends not only on chemical interactions (e.g., what type of binding partner is present) but also on the force applied to it (Chan & Odde 2008, Elosegui-Artola et al. 2018).

When the clutch is engaged and actin filaments are coupled to point contacts, the forces generated within the GC are transmitted to the outside world. As actin can no longer flow easily, the polymerization force, F_P, of F-actin pushes the cell membrane forward, leading to GC advance (Renkawitz et al. 2009). For example, the average peak protrusive forces of retinal ganglion cell GCs generated this way are on the order of 100 pN (Fuhs et al. 2013).

At the same time, the contractile forces, F_C, generated by myosin motors now pull the GC center toward the point contacts in the GC periphery (Betz et al. 2011). The resulting pulling on the point contacts leads to traction forces, F_T, acting on the environment. GC traction forces are sufficient to visibly deform environments as soft as brain tissue (Betz et al. 2011, Franze et al. 2009) and predict GC advance (Athamneh et al. 2015). They can be measured using, for example, traction force microscopy. Traction stresses (with the stress equal to force/unit area) of neuronal GCs are on the order of 10 Pa = 10 pN/μm^2 for neurons of the central nervous system (CNS) and ∼40 Pa for those of the peripheral nervous system, which are thus stronger than their CNS counterparts (Koch et al. 2012).

As neither F_P nor F_C leads to large movements of actin filaments when actin is engaged with point contacts, retrograde actin flow rates are decreased (Lin & Forscher 1995, Nichol et al. 2016).

In line with this observation, the sum of GC advance and retrograde flow velocities is often relatively constant (i.e., the faster the GCs advance, the slower are the retrograde flow rates) (Lin & Forscher 1995), although GC stimulation by, for example, serotonin can lead to simultaneous increases in retrograde flow rates and GC migration (Zhang et al. 2019), likely through the parallel regulation of actin polymerization and myosin-based contractility.

This system provides an efficient way to quickly regulate traction forces and GC advance simply through adjusting the level of clutch engagement (Chan & Odde 2008, Elosegui-Artola et al. 2018). Hence, force transmission through molecular clutches is dynamic; forces within GCs fluctuate in space and time (Betz et al. 2011, Koch et al. 2012, Polackwich et al. 2015).

Of course, in addition to F-actin and myosin, many other regulatory proteins associated with the actin cytoskeleton, including cross-linking, nucleating, capping, or severing proteins, as well as invading microtubules, may fine-tune the force balance in the GC by modulating the mechanical properties of the actin network (and thus force generation and propagation) (Cammarata et al. 2016, Omotade et al. 2017). However, in the end, GC motion is solely determined by the final force balance regardless of the individual components adding to this balance (Miller & Suter 2018) (see Section 4).

To allow continuous GC motility, the system relies on a constant turnover of F-actin and adhesion sites (Small et al. 2002). Actin filaments need to be disassembled in the GC center in order to provide actin monomers for further polymerization in the periphery and to enable further backward motion of actin filaments. Similarly, point contacts need to be strong enough to provide the traction required for traction force generation and GC advancement but also dynamic enough to be disassembled when the GC has advanced, as the GC would otherwise get stuck.

In summary, actin in GCs is constantly polymerized in the periphery and depolymerized in the center, while myosin motors pull the actin filaments toward the GC center or the GC center toward the point contacts in the periphery, depending on the level of (highly regulated) clutch engagement. At low clutch engagement, retrograde actin flow rates are high (up to ∼10 μm/h) and GCs do not advance much. With increasing clutch engagement, retrograde actin flow rates usually decrease, traction forces increase, actin polymerization pushes the membrane forward, and GCs advance at rates of usually up to a few tens of micrometers per hour (Mason & Erskine 2000, Roossien et al. 2013). Hence, clutch engagement offers a powerful regulatory switch between GC stalling and advance, enabling fast adaptation to changes in the environment.

Adding all the traction forces across the entire area of an advancing GC results in a finite, nonzero force value (**Figure 1*b***). This net force, F_N, is a contractile force that pulls the GC center toward the point contacts in the periphery at the same time as it pulls on the axon. Note that the magnitude of F_N does not necessarily depend on the magnitude of the total traction stress exerted by a GC, as local traction forces can cancel each other if they point in opposite directions (Hyland et al. 2014). Large traction stresses may indeed lead to an increase in GC adhesion, slowing down its migration and axon growth, while increases in F_N should lead to enhanced axon growth (see Section 4).

3. FORCES GENERATED IN AXONS

The cytoskeleton in mature axons has a very specific arrangement that is different from that in the GC. Growing axons are predominantly filled with microtubules; neurofilaments are added later when the axon matures. Within axons, most microtubules point with their fast-growing plus ends toward the GC. Microtubules have a persistence length of several hundred micrometers (Pampaloni et al. 2006) and thus can be considered rigid rods within cells. Hence, the addition of new tubulin dimers to growing microtubule ends leads to compressive (i.e., pushing) forces of up to ∼3–5 pN per microtubule (Dogterom & Yurke 1997, Laan et al. 2008) where microtubules

polymerize against an obstacle such as the GC cytoskeleton (Janson et al. 2003), similar to when actin polymerization in GCs leads to compressive forces acting on the cell membrane.

At the same time, molecular motors such as dynein may slide at least shorter microtubules apart (Athamneh et al. 2017, Craig et al. 2017, He et al. 2005, Roossien et al. 2014, Wang & Brown 2002), generating additional compressive forces pushing against the GC base (del Castillo et al. 2015, Dennerll et al. 1988, Jakobs et al. 2015, Kapitein & Hoogenraad 2015, Lu & Gelfand 2017, Lu et al. 2013). Depending on the number of microtubules in the axon cross section, the lengths of microtubules, the motor density, and the stall force of the motors, this sliding force has been estimated to be on the order of hundreds of piconewtons to a few nanonewtons (Jakobs et al. 2015, Roossien et al. 2014). Overall, microtubule network dynamics thus leads to compressive forces, which are dominated by microtubule sliding, pushing axons apart.

However, when neurites are slackened in vitro, or when their GCs' adhesion to the substrate is depleted, neurites contract (Bray 1979; Dennerll et al. 1988, 1989; Heidemann et al. 1995; Joshi et al. 1985; Recho et al. 2016), suggesting that, overall, neurites are under tension. By pulling on neurites, for example using calibrated glass microneedles, the axonal tension F_A has been estimated to be on the order of hundreds of piconewtons to a few nanonewtons (Athamneh & Suter 2015, Dennerll et al. 1988, Garate et al. 2015, O'Toole et al. 2015). Given that axons in a fully connected network (in which no GCs no longer exist) are under tension in vitro (Smit et al. 2017) as well as in vivo (Rajagopalan et al. 2010, Xu et al. 2010), this tension must be both intrinsically generated and at least partly independent of the pulling GC.

Axonal tension has been shown to be mediated by actomyosin-generated contractile forces (Bernal et al. 2007, Dennerll et al. 1988, Fan et al. 2017, Gallo 2006, Joshi et al. 1985, Mutalik et al. 2018, Tofangchi et al. 2016, Wylie & Chantler 2003). However, exactly how the interaction of actin and nonmuscle myosin II generates the contractile forces in neuronal processes is currently not fully understood (Dubey et al. 2018). Actin in axons is arranged as a regular pattern of rings surrounding the microtubules, which are spaced by spectrin tetramers (Xu et al. 2013) (**Figure 2**).

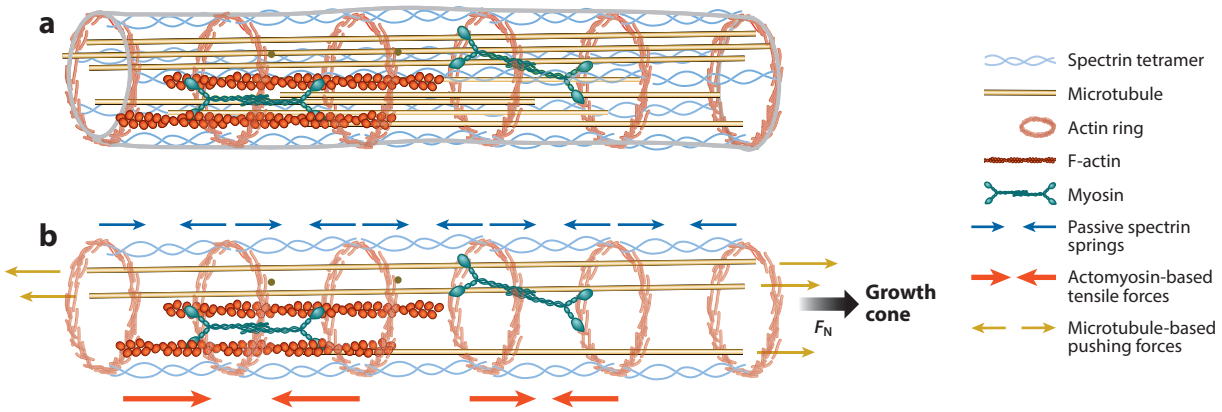

Figure 2

The origin of forces in neuronal axons. (*a*) Schematic drawing of the axonal cytoskeleton. Axons are filled with microtubules that, depending on the axons' developmental state, predominantly point with their plus ends out. Actin is mostly found as static rings arranged perpendicular to the axon axis and spaced by spectrin tetramers. In addition to these actin rings, focal actin hot spots and longer actin trails are also present. (*b*) Schematic illustration of the reduced cytoskeletal and axonal forces. Microtubule polymerization and sliding lead to pushing forces (*yellow arrows*). Actomyosin-based contractility leads to tensile forces along the axon (*red arrows*). Spectrin tetramers might act as passive springs (*blue arrows*), counteracting the pulling net force, F_N, from the growth cone (*black arrow*) and the microtubule-based pushing forces. In sum, forces generated within the axon, F_A, are contractile in nature.

In addition to this rather static array of actin rings, dynamic actin assemblies are found within axons, including focal actin hot spots and longer actin trails (up to ~9 μm) (Ganguly et al. 2015, Papandreou & Leterrier 2018).

There are about 190 nm between actin rings in axons (Leterrier et al. 2017, Xu et al. 2013). As non-muscle myosin II filaments are ~325 nm long (Beach et al. 2014, Billington et al. 2013), they could connect neighboring actin rings and thus pull them toward each other, leading to the buildup of the experimentally observed tension along the axon (Bernal et al. 2007, Dennerll et al. 1988, Fan et al. 2017, Gallo 2006, Joshi et al. 1985, Mutalik et al. 2018, Tofangchi et al. 2016, Wylie & Chantler 2003). This would explain why myosin shows a similar periodicity of 190 nm, which is, however, shifted out of phase with respect to actin rings (Berger et al. 2018). Myosin filaments could thus be arranged at an angle along axons, consequently fitting inside the gaps between neighboring actin rings and applying axial as well as circumferential tension (de Rooij et al. 2018, Fan et al. 2017, Miller & Suter 2018) (**Figure 2**).

The mechanical role of spectrin is currently less well understood. In red blood cells, the submembranous actomyosin cortex found in most other animal cell types is replaced by a spectrin network, which renders red blood cells very soft and elastic. Recent data suggest that spectrin is important for the maintenance of axons' structural integrity; spectrin tetramers could serve as some kind of passive elastic springs, counteracting the compressive forces generated by polymerizing and sliding microtubules (Krieg et al. 2017) and the pulling of the GC (**Figure 2**).

Overall, the pushing of microtubules is counterbalanced by tension (i.e., pulling forces) generated along axons. Actomyosin-generated tension is generally larger than microtubule-based compression. Hence, when adding microtubule and actomyosin dynamics–based forces as well as spectrin's passive springlike properties, the resulting total axonal force, F_A, is tensile in nature and acts in the opposite direction to the tensile (net) force, F_N, exerted by the GC (**Figure 3**).

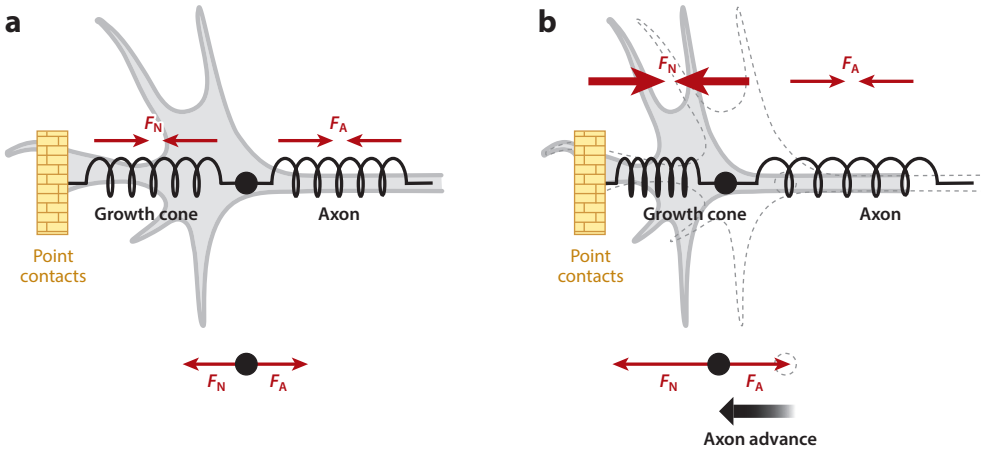

Figure 3

Force balance in axon growth. In both the growth cone (GC) and the axon, contractile forces (illustrated as *springs*) are dominant. The total tension along the axon, F_A, is relatively constant, while GC forces are dynamic and tightly regulated. The GC is attached to its environment by point contacts in the periphery. The net force, F_N, pulls the GC base toward the point contacts, and the GC thus pulls on the axon. (*a*) If the total contractile force exerted by the GC is balanced by axon tension, $F_N = F_A$, the GC stalls, and axon length does not change. (*b*) If $F_N > F_A$, the GC base advances, thus freeing up space for microtubules to grow into, and the axon extends.

4. THE COMBINATION OF FORCES LEADS TO WELL-CONTROLLED AXON GROWTH

How do GC and axonal forces combine to control the rate of axon growth? Axons grow by mass addition, mostly through increases in microtubule mass, amount of cell membrane, and the number of cell organelles (Athamneh et al. 2017); axon growth rates are limited by how fast microtubules can advance. However, does this mean that axon growth is driven by pushing forces of microtubules (Section 3), or is pulling of the advancing GC (Section 2) the main driver of axon growth? There is experimental evidence for either viewpoint.

In support of a dominant role for microtubule-based pushing forces, actin depletion in neurons leads to faster axon growth (Bradke & Dotti 1999), and when microtubules were moderately stabilized by taxol, and thus compressive forces were increased, axon growth and regeneration were enhanced (Hellal et al. 2011, Witte et al. 2008). These data suggest that actin-based contractility counteracts axon growth, and growth might therefore be driven by microtubule-based pushing forces (Witte et al. 2008).

In a seminal study, Steve Heidemann and colleagues (Lamoureux et al. 1989) lifted neuronal somata from the substrate using calibrated microneedles while the GC remained attached to the substrate and observed axon growth. If microtubule pushing were the driver of axon growth, the needle should have been deflected away from the advancing GC. However, as axons kept growing, the needle was reproducibly pulled toward the GC rather than pushed away from it, arguing that actomyosin-mediated contractile forces are the dominant driver of axon growth.

How can these apparently contradictory experimental results be explained? In a nutshell, in the presence of contractile forces, tension dominates (Lamoureux et al. 1989) and microtubule-based compressive forces are negligible. For example, when GCs pause, microtubule advance continues for a while and microtubules accumulate in the stalled GCs rather than pushing GCs forward (Tanaka & Kirschner 1991). Only after perturbations of the actomyosin cytoskeleton, when contractile forces are diminished and the GC actomyosin network is no longer in the way of microtubules, do microtubules become the dominant contributors to the local force balance. In the absence of contractile forces, F_N is negligible and the direction of F_A is reversed; it is now dominated by compressive microtubule-based forces. Hence, microtubules can easily push (i.e., grow) into the GC. This in turn allows axons to grow quickly, although in a less controlled manner (see Section 5). In this case, microtubule advance is the only factor limiting axon advance rates, which are thus at maximum.

After adding up all force components in an unperturbed system, both the total axonal force, F_A, and the net force, F_N, generated in the growth cone are contractile in nature and act in opposite directions—both pull on the GC center (**Figure 3**) (O'Toole et al. 2015). It is the balance between these forces that determines how an axon moves (de Rooij et al. 2018, Miller & Suter 2018, Recho et al. 2016): if $F_N > F_A$, axons advance; if $F_N = F_A$, axons stall; and if $F_N < F_A$, axons retract (Miller & Suter 2018, Recho et al. 2016).

Growth can thus be promoted by enhancing F_N, by decreasing F_A, or by diminishing actomyosin-based contractility in both GCs and axons, thus decreasing the resistance to microtubule advance (i.e., microtubule-based pushing forces are larger than the opposing forces). In an unperturbed environment, however, tension along the axon is rather constant (Hyland et al. 2014), and hence control over this force balance is largely achieved by regulating the net force, F_N. The stronger the GC pulls on the axon, the faster the axon grows, which is analogous to in vitro experiments in which axon growth rates increased linearly with the amount of externally applied forces (Dennerll et al. 1989, Fass & Odde 2003, Raffa et al. 2018).

In a simplified picture, the dense actin cytoskeleton in GCs prevents most microtubules from entering from the axon (Forscher & Smith 1988), likely because of steric hindrance (i.e., crowding),

as microtubule-based pushing forces are too weak to work against axon tension and the retrograde actin flow. Occasionally, a few microtubules may splay into the GC and help to reinforce or stabilize the growth direction, although changes in directional outgrowth are initially mediated mostly by the actin cytoskeleton (see Section 5). If F_N is high, the GC center is pulled toward the force-bearing point contacts at the periphery (see Section 2), which generates space for microtubules to invade. Once microtubules have grown into the GC base, they are stabilized in a process called engorgement (Dent et al. 2011).

The more the GC pulls on the axon, the more space is generated into which microtubules can grow, and the faster the axon extends. Hence, in this scenario axon rates of advance are limited by GC-mediated contractile forces and are less than the maximum microtubule rates of advance. This explains why perturbations of actomyosin contractility lead to enhanced axon growth rates (Blanquie & Bradke 2018), and why in healthy neurons the rate of microtubule advance is similar to that of the GC (Athamneh et al. 2017). As axons are viscoelastic and usually coupled to the surrounding extracellular matrix and/or other cells, the applied tension is dissipated with increasing distance from the GC (O'Toole et al. 2008), and thus most microtubule advance and hence axon growth occur near the advancing GC.

Of course, actin polymerization and cell adhesion through point contacts play an important role in regulating axon growth as well (O'Toole et al. 2008). Actin polymerization in the GC periphery pushes the membrane forward, leading to GC advance. In the periphery, new point contacts need to be assembled and be made strong enough to enable the buildup of contractile and polymerization forces. Once the GC has advanced, the GC base has been pulled toward the point contacts, and microtubules have invaded, older point contacts need to be disassembled, as otherwise GCs would get stuck.

Adhesion that is too strong would prevent both axon retraction (if adhesive forces are larger than F_A, regardless of F_N) and axon growth (as microtubules would not be provided with space into which they can grow), leading to GC stalling (Recho et al. 2016). If, however, adhesive forces are weaker than F_A, as, for example, after overactivation of myosin II by lysophosphatidic acid (LPA), axons retract in cultures on contractility-promoting stiff tissue culture plastics (Jalink et al. 1994) (cf. Section 6). In contrast, when neurons are cultured on soft substrates, which lead to reduced contractility (Koch et al. 2012), axons exposed to LPA do not retract but grow faster (Nichol et al. 2019), likely because F_N and F_A are increased but F_A does not exceed adhesive forces. Adhesion at optimum levels provides sufficient traction to facilitate GC advance, while an increase in traction force could overcome this adhesion and lead to GC retraction, a mechanism exploited in repulsive axon guidance (see Section 5).

At first glance, the force balance between an axon and GC may appear rather complicated. However, its regulation is largely confined to control of the contractility of the GC, which provides a straightforward way to alter axon growth by interfering with GC traction forces. GC-wide changes in contractility lead to changes in axon growth rates, while asymmetrical, local changes in GC traction forces redirect axon growth, which is exploited in axon pathfinding.

5. FORCES IN CHEMICAL AXON GUIDANCE

As discussed in Section 4, axon growth is driven by microtubule polymerization into the GC. Microtubules are rather stiff polymers (Pampaloni et al. 2006), which, in the absence of external constraints, grow more or less straight over relatively large distances (tens to hundreds of micrometers). Hence, if growing axons need to turn, they must force microtubules to bend, which requires forces that overcome an energy barrier (i.e., a threshold). Where do these forces come from?

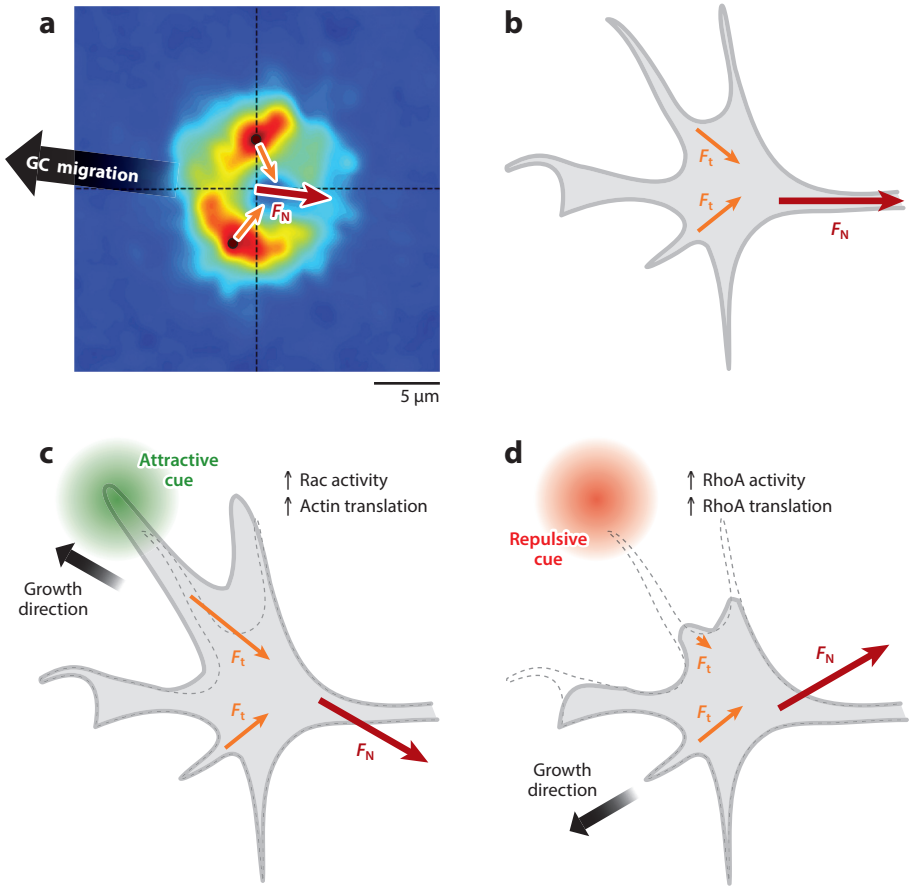

Figure 4

Forces in chemical axon guidance. (*a*) A time-averaged traction force map that shows growth cone (GC) migration toward nine o'clock (i.e., left). The largest traction forces (*red*) are exerted in the periphery at either side of the GC. Panel *a* adapted from Polackwich et al. (2015). (*b*) If these forces are similar in magnitude, the resulting net force, F_N (*red arrow*), has a near-zero component perpendicular to the growth direction and the axon grows straight. (*c*) In the presence of a gradient of an attractive chemical guidance cue, local actin polymerization is increased nearest to the source (e.g., through an increase in actin translation and Rac activity), GCs become asymmetrical, and traction forces on that side likely increase, leading to a change in the direction of F_N and thus a change in axon growth direction toward the signal. (*d*) In contrast, in the presence of a repulsive gradient, GCs collapse on the near side of the gradient (e.g., through an increase in contractility above the level of adhesive forces via RhoA activation and translation and subsequent actin depolymerization, and through local regulation of cell adhesion molecules), leading to an asymmetry in the GC and a change in the local force balance. In this case, traction forces farther away from the source of the chemical gradient dominate, and the axon is thus pulled away from the signal.

5.1. Cytoskeletal Basis of the Growth Cone Force Balance

In an elegant study in which traction forces exerted by growing neuronal GCs were recorded over time, Polackwich and colleagues (2015) showed that forces were mainly generated distally and laterally on both sites of the GC (**Figure 4*a***). The average of these forces was very similar on both sides and adding all traction force vectors across the GC led to a near-zero component of the net force, F_N, perpendicular to the current growth direction (**Figure 4*b***), resulting in straight

growth. Any change in this force balance leads to a change in the direction of F_N (**Figure 4*c*,*d***) and hence of axon tension. As early as the late 1970s, Dennis Bray (1979) nicely demonstrated, by pulling with a microneedle on an axon like a pick on a guitar string, that a change in the direction of axon tension is sufficient to change its growth direction.

In the current view, axons are guided by gradients of attractive and repulsive guidance cues, including netrins, semaphorins, slits, and ephrins, which lead to rapid asymmetrical remodeling of the traction force–producing actin cytoskeleton in GCs (Henley & Poo 2004, Piper et al. 2007, Sperry 1963, Tessier-Lavigne & Goodman 1996, Wen & Zheng 2006). Any asymmetry between the force centers on either side of the GC results in an imbalance in the local tension within the GC. If the resulting F_N is not aligned with the previous growth direction, the GC base is pulled toward the side of higher tension, and with it the space into which microtubules can invade (**Figure 4**). This way, the actomyosin cytoskeleton forces a directional change upon microtubule polymerization and thus axon growth.

This explains why depletion of actin with drugs such as cytochalasin may lead to enhanced axon growth but makes axons insensitive to chemical guidance cues in vitro as well as in vivo (Dent et al. 2011). In the absence of an intact GC actomyosin cytoskeleton, traction forces cannot be generated ($F_N \sim 0$) and locally modified in response to guidance cues. Thus, while microtubules can grow and slide into the leading tip of the axon without much resistance, they can only grow straight and can no longer be forced to change direction.

Filopodia, which are fingerlike protrusions of the GC packed with actin bundles, have been suggested to play an important role in axon pathfinding (Chien et al. 1993). However, at least in the absence of signaling molecules, forces generated by filopodia are significantly smaller than those generated by the GC's lamellipodium (Chan & Odde 2008, Koch et al. 2012), suggesting that filopodia are unlikely to be primarily involved in pulling the axon toward a new direction. However, filopodia significantly enlarge the surface area and effective radius particularly of small GCs in a three-dimensional environment, thus facilitating the detection of signaling molecule gradients. Furthermore, filopodia may be reinforced by the addition of actin and/or microtubules in response to guidance cues, and they may then also enhance their force production.

In addition, microtubules, which occasionally may splay into the GC (**Figure 1**), facilitate axon turning in response to guidance cues (Buck & Zheng 2002, Gordon-Weeks 2004), probably by consolidating and reinforcing the new growth direction determined by the GC's actomyosin cytoskeleton. Overall, cytoskeletal dynamics is regulated by a myriad of cytoskeleton-associated proteins, which may also couple microtubules to F-actin (Lowery et al. 2013).

5.2. Chemical Regulation of the Growth Cone Force Balance

Most signaling cascades triggered by chemical guidance cues are rather complex. Different types of neurons respond to a variety of different guidance cues, and responses to the same guidance cue may differ between axons and dendrites (Polleux et al. 2000) as well as between the same axons at different developmental time points (Campbell et al. 2001). However, most intracellular guidance cue–activated signaling pathways converge on the GC cytoskeleton, and for axon pathfinding effectively only the local force balance in the GC matters, regardless of how it is generated.

As an example, Netrin-1 may act as an attractive guidance cue. When GCs are exposed to gradients of it in a two-dimensional culture, within minutes local protein synthesis of β-actin as well as actin polymerization increases asymmetrically at the near side of the GC (Campbell & Holt 2001, Leung et al. 2006). These increases lead to a local increase in GC size and likely also in traction forces on that site, resulting in a change in the direction of the net force, F_N, and hence in growth toward the source of Netrin-1 (**Figure 4*c***). In agreement with this hypothesis, exposure of

GCs to Netrin-1 leads to an increase in their exerted forces in vitro (Moore et al. 2012, Toriyama et al. 2013).

The opposite change in the local force balance occurs when growing neurons encounter a gradient of a repulsive guidance cue, such as Semaphorin 3A (Campbell et al. 2001, He & Tessier-Lavigne 1997, Kolodkin et al. 1993). In this case, the signal leads to an asymmetrical GC collapse, likely reducing the traction forces on the near side of the GC and shifting the force balance toward the side that is farthest away from the repulsive cue (**Figure 4*d***).

As not only axon elongation but also axon turning is determined by the local force balance within the GC, actomyosin-driven contractile and F-actin-mediated polymerization forces are key players in axon guidance (Miller & Suter 2018, Smith 1988). Important upstream regulators of myosin activity and actin polymerization are the Rho GTPases RhoA, Rac1, and CDC42, and most guidance cues affect one or more of these Rho GTPases (Baba et al. 2018, Gallo 2006, Gallo et al. 2002, Jiang et al. 2015, Moore et al. 2012).

Attractive guidance cues often activate Rac1 and/or CDC42, which enhances local actin polymerization and the associated total polymerization force (**Figure 4*c***). Repulsive guidance cues, however, often activate RhoA, which enhances actomyosin contractility beyond the level of adhesion forces, leading to a local GC collapse and thus a decrease of traction forces near the source of the repulsive gradient (**Figure 4*d***). In agreement, inhibition of myosin II activity facilitates axon growth across inhibitory molecules (Hur et al. 2011). However, a local increase of GC contractility in combination with maintained adhesion would probably shift the force balance toward an attractive turning response, illustrating the complex interplay between forces and adhesion.

Hence, the control of cell adhesion is equally important for axon pathfinding. Accordingly, the link between F-actin and CAMs (i.e., point contact formation) is also regulated by chemical guidance cues (Nichol et al. 2016). For example, Netrin-1 activates Pak1, which phosphorylates the clutch molecule shootin1, which then couples F-actin to the adhesion molecule L1-CAM via the F-actin-binding molecule cortactin, in this way regulating the balance between retrograde flow rates and traction forces of the GC (Kubo et al. 2015, Toriyama et al. 2013). In all mechanisms mentioned in this section, chemical signals are transduced into well-regulated mechanical forces required for axon outgrowth.

6. MECHANICAL REGULATION OF AXON GROWTH AND GUIDANCE

As established in the previous sections, growing neurons exert forces on their environment and thus mechanically interact with it. These forces lead to the deformation of the soft environment, and the amount of deformation (and thus the traction the tissue provides) depends on its local mechanical properties, such as its stiffness. Much as we can bicycle faster on a paved road than along a sandy beach, the mechanical properties of a neuron's environment impact axon growth.

Seminal studies by Paul Janmey and colleagues (Flanagan et al. 2002, Georges et al. 2006) showed that, in vitro, neuronal growth indeed depends on the stiffness of the cell culture substrate, as spinal cord neurons branched more on soft substrates mechanically resembling CNS tissue. Further studies then revealed more substrate mechanics–dependent growth patterns of neuronal processes, which differed between different types of neurons, between axons and dendrites, and between the investigated species (Chan & Odde 2008; Jiang et al. 2008, 2010; Koch et al. 2012; Koser et al. 2016; Kostic et al. 2007; Nichol et al. 2019; Previtera et al. 2010), indicating that neuronal mechanosensitivity is well controlled and complex.

Koser and colleagues (2016) provided the first in vivo evidence for the regulation of axon growth by tissue stiffness. As in the bicycle analogy mentioned above, *Xenopus* retinal ganglion cell axons grew faster and bundled more on stiffer substrates, and both softening of brain tissue

and perturbations of the mechanosensitive ion channel Piezo1 led to severe pathfinding errors in vivo, with reduced axonal fasciculation (bundling) and growth. Moreover, local stiffness gradients in developing brain tissue were shown to contribute to the regulation of axon pathfinding (Koser et al. 2016, Thompson et al. 2019).

6.1. Mechanical Properties of Central Nervous System Tissue

The mechanical properties of CNS tissue can be quantified in terms of its shear modulus G, which is—depending on the region, age, gender, and method used to measure it—usually somewhere in the range of tens to hundreds of Pascals (equal to piconewtons per square micrometer). Importantly, brain and spinal cord tissue mechanics appears to be precisely regulated. While CNS tissue stiffness is highly heterogeneous in space and time, its local mechanical properties follow clearly defined, reproducible patterns (Christ et al. 2010, Elkin et al. 2007, Iwashita et al. 2014, Koser et al. 2015, Moeendarbary et al. 2017, Thompson et al. 2019). Mechanical CNS tissue properties are dynamic, and in the case of the developing *Xenopus* brain can drastically change within several tens of minutes (Thompson et al. 2019). Furthermore, advancing GCs can extend F-actin-rich invadosomes, which may remodel the ECM by secreting metalloproteases, in this way altering mechanical tissue properties in the immediate environment and facilitating axon growth (Santiago-Medina et al. 2012).

During early development, when there is usually little ECM found in the CNS, local tissue stiffness is largely regulated by the density of cell bodies: the denser the cells are packed, the stiffer the tissue (Koser et al. 2016, Thompson et al. 2019). At later stages, other factors, such as the degree of myelination and the amount of different parts of the ECM, increasingly contribute to tissue mechanics (Hassan et al. 2019, Koser et al. 2015, Moeendarbary et al. 2017, Segel et al. 2019, Weickenmeier et al. 2016).

6.2. How Growing Axons Measure the Mechanical Properties of Their Environment

Generally, the function of a protein is determined by its structure. Any protein that is exposed to forces strong enough to alter its shape may experience changes in its function. While this mechanosensitivity may be coincidental for some proteins—for example, NMDA receptors have been shown to be activated by shear stress, inducing Ca^{2+} entry into astrocytes (Maneshi et al. 2017), although it is currently not clear if this behavior is connected to a physiological function—mechanotransduction, i.e., the conversion of a mechanical signal into an intracellular chemical signal, is a main function of some other proteins.

There are numerous proteins with primary mechanical functions that may respond differently to the same mechanical stimuli. Also, the same proteins may respond differently to mechanical signals depending on the context; GC lamellipodia and filopodia respond in opposite ways to substrate stiffness using similar molecular mechanotransducers.

Potential neuronal mechanotransducers include proteins located at point contacts, the critical interface at which intracellularly generated forces are transmitted to the outside world, including talin and vinculin (Kerstein et al. 2013, Renaudin et al. 1999). At least in focal adhesions of other cell types, talin directly links integrins to actin filaments, and vinculin reinforces this link by coupling talin to actin. Using FRET-based intracellular force sensors, it was shown that in migrating cells both talin and vinculin are indeed under significant tension (Grashoff et al. 2010, Ringer et al. 2017).

The application of forces to talin in vitro exposes cryptic binding sites for vinculin (del Rio et al. 2009). Hence, when GCs exert strong enough forces on their environment, talin is likely put

under tension and vinculin-binding sites are exposed. Vinculin is then recruited to talin, reinforces the link between talin and actin, and thus strengthens the engagement of actin with the clutch.

An increase of GC forces beyond the critical threshold for clutch reinforcement through vinculin can be achieved by chemical signaling (e.g., through activation of RhoA). However, the mechanical properties of a GC's environment also regulate GC forces. As stiffer substrates resist deformation more than softer substrates, forces build up more quickly on stiffer substrates. If the forces are transmitted to the environment faster than the lifetime of the integrin-ECM bond, talin unfolds, vinculin binds, and the clutch gets reinforced, allowing it to build up even larger forces. However, when the force transmission rate is slower than the lifetime of the integrin-ECM bond (on softer substrates), the bond dissociates before talin can be unfolded (Elosegui-Artola et al. 2016).

This model suggests that on stiffer substrates more talin molecules experience forces above the critical threshold, leading to enhanced vinculin recruitment to previously cryptic binding sites, which causes forces to increase further. Thus, the regulation of GC forces by substrate stiffness leads to the activation of a positive feedback loop: Forces above a certain threshold lead to the reinforcement of the clutch and thus to even larger forces. Point contacts thus mature faster and become stronger on stiffer substrates than on softer ones (Elosegui-Artola et al. 2016). This strengthening of the clutch results in reduced retrograde flow and larger traction forces (see Section 2), which again lead to more stretching of talin, vinculin recruitment, and so forth, until all binding sites of talin for vinculin are saturated (Atherton et al. 2015, Ciobanasu et al. 2014) and traction forces reach a substrate stiffness–dependent maximum (Koch et al. 2012).

This process is analogous to how chemically induced increased GC contractility promotes the formation and stabilization of point contacts (Woo & Gomez 2006). The talin-vinculin-based force-sensitive mechanism, leading to stronger forces on stiffer substrates (Koch et al. 2012), thus not only relays but also amplifies a mechanical signal—substrate stiffness—and could thus be considered an equivalent to a second messenger found in metabotropic signaling pathways.

As a direct consequence of the increased clutch engagement in GC lamellipodia on stiff substrates, GCs pull more strongly on their axons (Koch et al. 2012), which could directly increase axon growth rates (Section 4). In agreement with this hypothesis, axons of *Xenopus* retinal ganglion cells grow faster on stiffer substrates (Koser et al. 2016).

Furthermore, in filopodia the level of engagement of the actin cytoskeleton with point contacts is directly regulated by substrate stiffness via a motor–clutch mechanism, although the relationship is inverted compared to that in the lamellipodium. Here, retrograde flow rates are slower and traction forces stronger on softer substrates when compared to stiffer substrates (Chan & Odde 2008). This was explained by a rather weak coupling of point contacts to actin filaments, so that the clutch cannot resist large forces, leading to frictional slippage on stiffer substrates; this explanation would indicate that there are structural differences between point contacts in lamellipodia and filopodia. This would in turn explain why GCs on stiffer substrates usually have more dominant lamellipodia and smaller filopodia than do those on softer substrates (Koser et al. 2016). Also, these differences suggest that filopodia might be optimized to explore softer tissues, while GC-mediated axon growth works better in stiffer tissues. In line with this hypothesis, tissue along which *Xenopus* retinal ganglion cell axons grow in vivo is stiffer than their target, the optic tectum, where GCs slow down and explore their environment to find their partners (Koser et al. 2016).

In addition to the structural proteins talin and vinculin, signaling proteins such as focal adhesion kinase (FAK) are also part of point contacts (Renaudin et al. 1999). FAK activity in neurons has been shown to be regulated by substrate stiffness (Jiang et al. 2008, Moore et al. 2012, Robles & Gomez 2006). Furthermore, proteins located outside of point contacts may be directly involved in mechanotransduction as well (for a recent overview about mechanosensitive proteins in GCs,

see Kerstein et al. 2015). As an example, mechanosensitive ion channels are involved in the regulation of a diverse set of functions in different types of neurons in different species (Chen et al. 2018, Franze et al. 2009, Kerstein et al. 2013, Koser et al. 2016, Qiu et al. 2019, Song et al. 2019), and at least ion channels of the Piezo family have no known ligands in vivo (Coste et al. 2010, 2012), suggesting that their activity is purely regulated by mechanical signals.

After mechanical activation of these signaling proteins, important downstream effectors include calcium ions and RhoA (Franze et al. 2009, Kerstein et al. 2013, Nichol et al. 2019). Both of these effectors act on, among others, the actomyosin cytoskeleton, and both therefore have critical roles in regulating axon growth and pathfinding (Gomez & Spitzer 1999, Hong et al. 2000, Hu et al. 2001, Yuan et al. 2003) (see also Section 7).

Notably, not only forces generated in the GC but also tension along the axon may play an important role in regulating axon development. Depending on the coupling of the cytoskeleton to the cell membrane and potentially on other factors, the amount of tension generated by the cytoskeleton may directly or indirectly impact membrane tension in the GC and along the axon shaft, which at least in vitro has been shown to regulate gating of the mechanosensitive ion channel Piezo1 (Lewis & Grandl 2015).

Furthermore, during early neuronal development, most axons do not grow individually but instead grow together along a few pioneer axons (Bentley & Caudy 1983). As a result, axons usually grow in bundles—they fasciculate. For a long time it was assumed that axon fasciculation is controlled at the level of the GC. Recently, however, it was shown that axons can bundle and unbundle far away from and independent of the GC, and that the amount of fasciculation is regulated by axon tension (Šmít et al. 2017).

Finally, note that, for many neurons, the distance covered by GC-mediated axon growth only corresponds to a fraction of the axon's final length. Once the GC reaches its target during early development, synapses are formed, and the GC disappears. Subsequently, the body keeps growing, and axon elongation continues in a process termed towed growth. In this process, first suggested by Paul Weiss (1941), the growing body pulls on axons similarly as GCs did before, thus providing space into which microtubules can grow and deliver material required for growth to the periphery. The first in vitro evidence for towed growth came from a seminal study by Dennis Bray (1984), showing that GC-independent axon growth can be stimulated by the external application of mechanical tension. As mentioned in Section 4, axon growth rates increase linearly with applied forces (Dennerll et al. 1989, Fass & Odde 2003, Pfister et al. 2004, Raffa et al. 2018). An elegant recent study has shown the first in vivo evidence for towed axon growth in the developing zebrafish brain (Breau et al. 2017).

7. INTEGRATING CHEMICAL AND MECHANICAL SIGNALS IN NEURONAL DEVELOPMENT AND DISEASE

As many of the downstream effectors of mechanotransduction are shared with chemical signaling pathways, there is very likely cross talk between them that allows neurons to integrate chemical and mechanical signals. Hence, local tissue stiffness and cellular forces might impact the response of a neuron to a chemical signal, while at the same time chemical signals may change local mechanical tissue properties and/or the neuronal perception of tissue stiffness.

For example, as mentioned in Section 6, RhoA activity in neurons is controlled by substrate stiffness (Nichol et al. 2019), with higher substrate stiffness leading to increased RhoA activity, although the exact mechanism remains to be determined. At the same time, neuronal guidance cues may also regulate the activity of RhoA (Jiang et al. 2015; Moore et al. 2009, 2012) (Section 5). Repulsive signaling by Semaphorin 3A, for instance, enhances RhoA activity (Hu et al.

2001), while the inhibition of RhoA enhances axonal chemoattraction by Netrin-1 (Moore et al. 2008). Hence, both the stiffness of the surrounding tissue and its chemical composition impact RhoA signaling in GCs, which regulates actomyosin-based contractile forces and thus axon growth.

Consequently, stiffer brain regions, which cause an increase in neuronal RhoA activity, may enhance repulsive chemical signaling, which is mediated, among others, by RhoA. In contrast, softer brain regions could attenuate repulsive signaling and enhance chemoattraction through decreased RhoA activity. While specificity of axon guidance has to arise from receptor-mediated (chemical) interactions between GCs and their environment, tissue mechanics may not only directly regulate axon advance rates (see Section 6) but also contribute to axon guidance by fine-tuning the response to chemical guidance cues and optimizing the signal-to-noise ratio.

This integration of chemical and mechanical signals is important not only for developmental but also for pathological processes. Many neurodegenerative diseases are accompanied by changes in CNS tissue mechanics (Gerischer et al. 2018, Lipp et al. 2018, Streitberger et al. 2012), and glial scars, which form after spinal cord injuries, are significantly softer than healthy tissue (Moeendarbary et al. 2017). Adult mammalian neurons cannot regenerate through these soft scars, and Rho GTPase signaling is involved in axon regeneration (Blanquie & Bradke 2018). Hence, these changes in the mechanical properties of brain and spinal cord tissue are likely to contribute to perturbed neuronal signaling and homeostasis, and any holistic approaches aiming to cure these conditions should take mechanical signaling into account.

8. CONCLUSIONS

Work over the past four decades has revealed that both chemical and mechanical signals are important for regulating axon growth and pathfinding. It is a subtle balance of intrinsically generated forces that determines where axons grow. While the axon itself is under constant tension, forces exerted by the GC are highly dynamic. The levels of force generation and force transmission are tightly regulated by both chemical and mechanical signals.

Rho family kinases are key players in the regulation of force generation in neuronal GCs (Woo & Gomez 2006). RhoA controls actomyosin-based contractility proximal to point contacts to pull the GC base forward and open space into which axonal microtubules can grow (**Figure 3**). Rac1 and CDC42, however, drive actin polymerization distal to point contacts to push the membrane forward. An asymmetry in Rho kinase activity across a GC results in an asymmetrical force distribution, a reorientation of the net force, F_N, and consequently in axon turning (**Figure 4**). There is cross talk between the Rac1, Cdc42, and RhoA pathways (Yuan et al. 2003), and all of them are regulated by neuronal guidance cues as well as by substrate mechanics.

Similarly, the buildup of point contacts and the engagement of molecular clutches are regulated not only by guidance cues (Baba et al. 2018, Toriyama et al. 2013) but also by the forces exerted by the GC (Buck et al. 2017, Chan & Odde 2008, Giannone et al. 2009). The GC-generated forces become stronger with increasing substrate stiffness (Koch et al. 2012).

There is intense cross talk between intracellular signaling pathways activated by chemical guidance cues and mechanical signals, and a change in any one of these signals potentially alters the response of the cells to the other. Future work will unravel more molecular details of mechanotransduction pathways in neurons, and thus identify more crossover points between chemical and mechanical signaling. The inclusion of mechanical interactions between neurons and their environment in our picture of CNS development and pathology will enable a better understanding of fundamental processes, which, in the future, might lead to breakthroughs in biomedical approaches related to neurodegenerative disorders and neuronal regeneration.

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